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INVITED REVIEW

Tetherin and Its Viral Antagonists

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Abstract Restriction factors comprise an important layer of host defense to fight against viral infection. Some restriction factors are constitutively expressed whereas the majority is induced by interferon to elicit innate immunity. In addition to a number of well-characterized interferon-inducible antiviral factors such as RNaseL/OAS, ISG15, Mx, PKR, and ADAR, tetherin (BST-2/CD317/HM1.24) was recently discovered to block the release of enveloped viruses from the cell surface, which is regarded as a novel antiviral mechanism induced by interferon. Here, we briefly review the history of tetherin discovery, discuss how tetherin blocks virus production, and highlight the viral countermeasures to evade tetherin restriction.

Keywords HIV-1 · Tetherin · Vpu · Virus release · Interferon

Introduction

Interferon triggers innate immunity to protect cells from viral infection. Production of interferon is initiated with recognition of viral components, mainly viral nucleic acids,

by cellular pathogen recognition receptors that are located on cellular membranes or within the cytosol (reviewed in Kawai and Akira 2006; Douville and Hiscott 2010). For example, membrane-associated Toll-like receptor 3 (TLR3) recognizes double-stranded viral RNA, recruits TIR domain-containing adaptor inducing IFN- β , and activates the interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathways to induce type I interferon production. TLR7 and TLR9 are both located in endosomes. After recognizing single-stranded viral RNA or double-stranded viral DNA, they recruit myeloid differentiation primary response gene 88 and activate the IRF7 and NF- κ B pathways. The cytosolic retinoic-acid-inducible gene I and melanoma differentiation-associated gene 5 recognize either double-stranded viral RNA or 5'-triphospho viral RNA and activate the MAVS (also known as IPS-1, VISA, and Cardif) signaling transduction pathway, which leads to interferon production.

Interferon binds to its receptor on the cell surface and activates tyrosine kinase 2 and Janus kinase 1 that in turn phosphorylates signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STAT1/STAT2 then translocates into the nucleus and forms the interferon-stimulated gene factor 3 (ISGF3) complex together with IRF9. The ISGF3 complex activates the expression of a wide spectrum of genes, collectively named interferon-stimulated genes (ISGs) that together establish an antiviral state. Among hundreds of ISGs, those with well-defined antiviral activities include RNaseL/OAS (2',5'-oligoadenylate synthetase), ISG15, myxovirus resistance (Mx), protein kinase R (PKR), and adenosine deaminase, RNA-specific (ADAR) that inhibit virus infection by different mechanisms (reviewed in Sadler and Williams 2008). Upon binding to double-stranded RNA, OAS

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oligomerizes and synthesizes 2',5'-oligoadenylates that bind to and activate RNaseL. The activated RNaseL degrades both cellular and viral RNA. ISG15 is conjugated to cellular and viral proteins to modulate cellular response and inhibit virus infection. This event is termed ISGylation that is catalyzed sequentially by three enzymes called ubiquitin E1 enzyme, E2-conjugating enzymes (such as UBC8), and E3 ligase enzymes (such as HERC5, homologous to the E6-associated C terminus domain and RCC1-like-domain-containing protein 5). Mx proteins are dynamin-like large GTPases. They inhibit the infection of a wide range of RNA and DNA viruses by binding to viral nucleocapsids or other viral components and subsequently trapping viral complexes as a result of Mx oligomerization. PKR is a kinase that is dormant in its monomeric form. Upon binding to viral double-stranded RNA, the kinase activity is activated, which leads to phosphorylation and dimerization of PKR. The activated PKR then phosphorylates EIF2 α and thus inhibits translation of cellular and viral proteins. ADAR is an adenosine deaminase that binds double-stranded RNA and converts adenosine to inosine. In addition to these antiviral effectors, recent studies discovered that an ISG named tetherin (also called BST-2/CD317/HM1.24, bone marrow stromal cell antigen 2) inhibits viral infection via a novel mechanism by blocking the release of nascent enveloped viral particles from the cell surface.

Discovery of tetherin as an antiviral protein

BST-2 (or HM1.24) was first reported as a protein that expresses at the surface of human plasma cell lines, bone marrow stromal cell lines and B cells, and is thus speculated to have a role in B-cell development (Goto et al. 1994; Ishikawa et al. 1995). Its potential role as a component of the host antiviral defense mechanism was suggested by its being a target of the K5 protein (also named MIR2) of Kaposi's sarcoma-associated herpesvirus (Bartee et al. 2006). The antiviral activity of BST-2 was subsequently demonstrated for its ability to inhibit the release of HIV-1 that is deficient in Vpu and was therefore given the name tetherin (Neil et al. 2008; Van Damme et al. 2008).

In addition to structural and enzymatic proteins including Gag, Pol, and Env, HIV-1 also encodes two regulatory proteins Tat and Rev that are essential for viral gene expression, as well as four accessory proteins named Vpr, Vif, Vpu, and Nef that play important roles in HIV-1 pathogenesis (reviewed in Anderson and Hope 2004; Malim and Emerman 2008). For example, Vif recognizes a cellular cytidine deaminase named apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 G (APOBEC3G), triggers polyubiquitination of APOBEC3G through recruiting the

Cullin5-containing E3 ligase, and sends APOBEC3G to proteasomes for degradation. In the absence of Vif, APOBEC3G associates with HIV-1 RNP complex, gets packaged into virus particles, and induces hypermutation of viral cDNA by converting cytidines into uridines during reverse transcription (reviewed in Henriet et al. 2009; Niewiadomska and Yu 2009). It has been known for quite a long time that Vpu is required for efficient HIV-1 production in certain cell lines such as HeLa and primary T cells but not in cells such as HEK293 and COS-7 (Strebel et al. 1989; Göttinger et al. 1993). The Vpu-deficient HIV-1 particles encounter a difficulty of leaving the surface of Vpu-non-permissive cells such as HeLa. A dominant cellular factor was speculated to underlie this restriction activity because the restriction phenotype was recreated in the HeLa/COS-7 heterokaryotic cells (Varthakavi et al. 2003). Subsequent studies revealed that this putative restriction factor is interferon-inducible (Neil et al. 2007). A long quest for this mysterious factor by several labs led to the discovery of its identity as tetherin, named after its function to tether nascent HIV-1 particles to the cell surface (Neil et al. 2008; Van Damme et al. 2008). After this finding, a variety of enveloped viruses were also shown to be subject to the restriction of tetherin. These include HIV-2 (Le Tortorec and Neil 2009), simian immunodeficiency virus (SIV) (Jouvenet et al. 2009), alpharetrovirus (Rous sarcoma virus) (Jouvenet et al. 2009), betaretrovirus (Mason-Pfizer monkey virus) (Jouvenet et al. 2009), gammaretrovirus (human T-cell leukemia virus type 1 (HTLV-1)) (Jouvenet et al. 2009), filoviruses (Marburg virus (Jouvenet et al. 2009; Sakuma et al. 2009) and Ebola virus (Jouvenet et al. 2009; Kaletsky et al. 2009)), arenaviruses (Lassa virus (Sakuma et al. 2009)), and herpesvirus (Kaposi's sarcoma-associated herpesvirus (KSHV) (Mansouri et al. 2009)). The two forthcoming questions are how tetherin retains enveloped viral particles to the cell surface and how viruses evade this restriction.

What is tetherin?

Human tetherin is encoded by a single copy of the bst-2 gene on chromosome 19p13.2 that is split into four exons (Ishikawa et al. 1995). This gene is relatively non-polymorphic with only one nonsynonymous single-nucleotide polymorphism that is listed in the human genome database and located in the coding region for the ectodomain of tetherin. Tetherin orthologs are present in the genomes of mammals but with significant sequence divergence (McNatt et al. 2009).

Tetherin is constitutively expressed in mature B cells, some cancer cell lines, bone marrow stromal cells, monocyte-derived macrophages, and plasmacytoid dendrit-

ic cells (pDCs) (Ishikawa et al. 1995; Vidal-Laliena et al. 2005; Blasius et al. 2006; Mitchell et al. 2009; Miyagi et al. 2009). Interferon strongly stimulates tetherin expression due to the presence of interferon response elements and a binding site for STAT3 in the promoter region of *bst-2* gene (Blasius et al. 2006). However, the level of tetherin expression as part of the interferon response appears to be cell-type-dependent (Miyagi et al. 2009). Regarding cancer T-cell lines, which are commonly used in laboratories to study HIV infection, the basal expression levels of tetherin vary but are all interferon-inducible (Neil et al. 2008; Van Damme et al. 2008; Rong et al. 2009).

When first described in B cells, tetherin was suggested to be a marker for B-cell maturation (Ishikawa et al. 1995). Its subsequent detection on multiple myeloma cells suggested a tetherin-based therapeutic strategy to induce cell-mediated cytotoxicity with tetherin antibody (Ozaki et al. 1997; Ono et al. 1999). In an epithelial cell line, tetherin interacts and modulates the subapical actin cytoskeleton, indirectly, via RICH2, EBP50, and ezrin (Rollason et al. 2009). It remains undetermined whether the cytoskeleton-modulating activity is involved in tetherin's function of restricting viral release. Tetherin is also a ligand for immunoglobulin-like transcript 7 (ILT7) on pDCs (Cao et al. 2009). Since ILT7-mediated signaling strongly inhibits production of interferon and other cytokines in pDCs (Brown et al. 2004; Cao et al. 2006), tetherin may negatively regulate interferon and cytokine production (Cao et al. 2009).

Tetherin localizes to the plasma membrane, the trans-Golgi network (TGN) and the early and recycling endosomes, and cycles between these membrane compartments (Kupzig et al. 2003; Habermann et al. 2010). Endocytosis of tetherin from the plasma membrane is clathrin-dependent and involves an interaction of tetherin with the adaptor proteins AP-1 and AP-2 (Rollason et al. 2007; Masuyama et al. 2009). In an epithelial cell line, tetherin localizes to the apical membrane following cellular polarization (Rollason et al. 2009). At the cell surface, tetherin localizes to lipid rafts (Kupzig et al. 2003; Rollason et al. 2007, 2009; Goffinet et al. 2009). Immunofluorescence microscopy reveals tetherin as punctual clusters at the cell surface (Hinz et al. 2010). HIV-1 Gag assembly and virus budding also occur at these membrane microdomains (Waheed and Freed 2009). In addition, lipid rafts are also involved in forming virological synapses and promoting cell-to-cell transmission of viruses (Popik et al. 2002; Piguet and Sattentau 2004; Popik and Alce 2004; Jolly and Sattentau 2005).

How does tetherin block virus release?

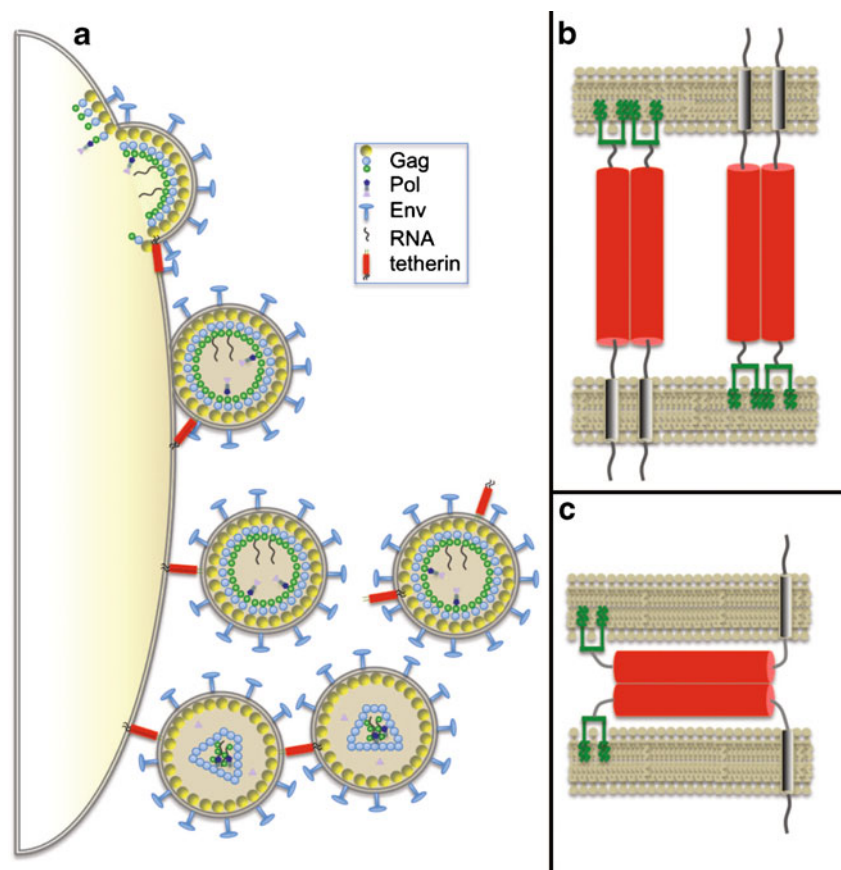
Tetherin retains nascent viral particles at the cell surface by linking them to the cellular membrane and to each other

(Fig. 1a). Electron microscopy shows accumulation of viral particles at the surface of tetherin-expressing cells (Neil et al. 2008; Perez-Caballero et al. 2009; Fitzpatrick et al. 2010). Tetherin is colocalized with viral Gag at the cell surface (Neil et al. 2008; Van Damme et al. 2008; Perez-Caballero et al. 2009) and is detected in the membrane of viruses that are released from the cell surface either spontaneously (Fitzpatrick et al. 2010; Habermann et al. 2010) or by shearing force (such as vortexing) (Miyagi et al. 2009). Results of immunoelectron microscopy further revealed that tetherin is located between the tethered viral particles and between the tethered virus particle and the plasma membrane, which indicates a direct role of tetherin in blocking virus release. This conclusion is further supported by two lines of evidence. First, human tetherin, when expressed in non-human cells, can restrict release of budding virions (Sato et al. 2009). Second and more importantly, the antiviral function of tetherin is, to a certain degree, independent of its sequence. An antiviral competent artificial tetherin has been reported, which has its transmembrane domain derived from transferrin receptor, coiled-coil domain from dystrophin myotonic protein kinase, and glycosylphosphatidylinositol (GPI) anchor from urokinase plasminogen activator receptor (Perez-Caballero et al. 2009). These results suggest that the antiviral activity of tetherin unlikely requires a co-factor.

Structural determinants of tetherin's antiviral function Tetherin is a type II transmembrane protein (Ishikawa et al. 1995; Kupzig et al. 2003). It bears a short cytoplasmic N-terminal region, a transmembrane region, an ectodomain, and a C-terminal GPI anchor (Kupzig et al. 2003). This topology is rare and only shared with an isoform of the prion protein (Hegde et al. 1998; Stewart et al. 2001). Tetherin forms disulfide-linked dimers. It is heterogeneously glycosylated and thus migrates as 30–36-kDa proteins on a sodium dodecyl sulfate–polyacrylamide gel. These structural features together determine tetherin's antiviral function and also regulate its sensitivity to different viral antagonists.

The short cytoplasmic domain (at amino acid positions 1 to 21) contains a conserved dual tyrosine motif (YXY_{6–8}) that is crucial for clathrin-mediated endocytosis through recruiting AP-1 and AP-2 adaptor proteins (Rollason et al. 2007). The two lysine residues (at positions 18 and 21) are ubiquitinated by the RING-CH K5 ligase of KSHV, which leads to tetherin degradation (Mansouri et al. 2009; Pardieu et al. 2010). Human tetherin lacks the 14-D/GDIWK-18 motif that constitutes the interaction site of Nef and is thus resistant to Nef-mediated antagonism (Jia et al. 2009; Zhang et al. 2009). Additionally, the cytoplasmic domain of tetherin has been described to indirectly modulate subapical actin cytoskeleton in polarized endothelial cells via RICH2, EBP50, and ezrin (Rollason et al. 2009).

Fig. 1 Antiviral mechanism of tetherin. **a** Tetherin localizes to the virus budding site and incorporates into the viral membrane. Tetherin exerts its antiviral action by tethering viral particles to the cell and to each other. Further, tetherin inhibits protease-mediated viral maturation of released viral particles. **b, c** Models of molecular tethering mechanism. Tetherin homodimers are likely oriented in parallel. Tetherin links viral particles to cells by insertion of one terminus into the virion membrane, while the other is located in the cellular membrane (**b**), or tetherin may link viral particles and cells via dimerization of the ectodomains of monomers with both termini inserted in either the virion membrane or the cellular membrane



The transmembrane region (at amino acid positions 22 to 43) consists of a single membrane-spanning helix. In the case of HIV-1, the transmembrane region renders human tetherin sensitive to Vpu-mediated downmodulation from the cell surface and subsequent degradation (Gupta et al. 2009a; McNatt et al. 2009; Rong et al. 2009). Several amino acid residues across the transmembrane domain of tetherin have been reported to regulate the sensitivity to Vpu and tetherin/Vpu interaction; these include L22, L23, G25, I26, V30, I33, I36, and T45 (Fig. 2) (Gupta et al. 2009a; McNatt et al. 2009; Rong et al. 2009). Interestingly, results of computer modeling of the structure of tetherin transmembrane domain show that these amino acid residues are positioned at one side of the helix, suggesting that they may together form the contact site with Vpu (Fig. 2).

The ectodomain (amino acids 44 to 160) assumes a long single α -helix as shown by the results of X-ray crystallography (Fig. 3) (Hinz et al. 2010; Schubert et al. 2010; Yang et al. 2010a). The complete ectodomain extends to a length of 150 to 170 Å which includes the 90-Å C-terminal coiled-coil domain (Hinz et al. 2010). Two tetherin molecules dimerize via this parallel disulfide-linked coiled-coil structure that is mainly stabilized by interactions throughout the two-third C-terminal portion of the ectodomain (Fig. 3). The N-terminal portion of ectodomain appears to be

relatively flexible at two hinges (positions A88 and G109) and mediates the tetramerization of two tetherin dimers by forming an antiparallel four-helix bundle (Fig. 3) (Hinz et al. 2010; Schubert et al. 2010). Although two tetherin dimers form a tetramer in crystals, mutants that are deficient in tetramerization retain most of the antiviral activity (Schubert et al. 2010; Yang et al. 2010a). The length of ectodomain is crucial for tetherin to block virus release, which suggests a molecular ruler function to keep the two membrane-spanning termini at a distance that is required for maximal antiviral activity (Hinz et al. 2010; Yang et al. 2010a).

The ectodomain of tetherin also contains three cysteines ($C_{53}C_{63}C_{91}$) that form disulfide bonds and therefore stabilize tetherin homodimers. In addition, there exist two sites for post-translational N-linked glycosylation ($N_{65}N_{92}$) (Ohtomo et al. 1999; Andrew et al. 2009). While glycosylation was shown to be dispensable for the antiviral function, formation of at least one disulfide bond is crucial for tetherin to block virus release (Andrew et al. 2009; Perez-Caballero et al. 2009; Hinz et al. 2010).

The C-terminal region of tetherin contains a signal for the addition of a GPI anchor. The nascent protein is predictably cleaved at a serine residue (position 160) in the endoplasmic reticulum (ER) followed by the attachment

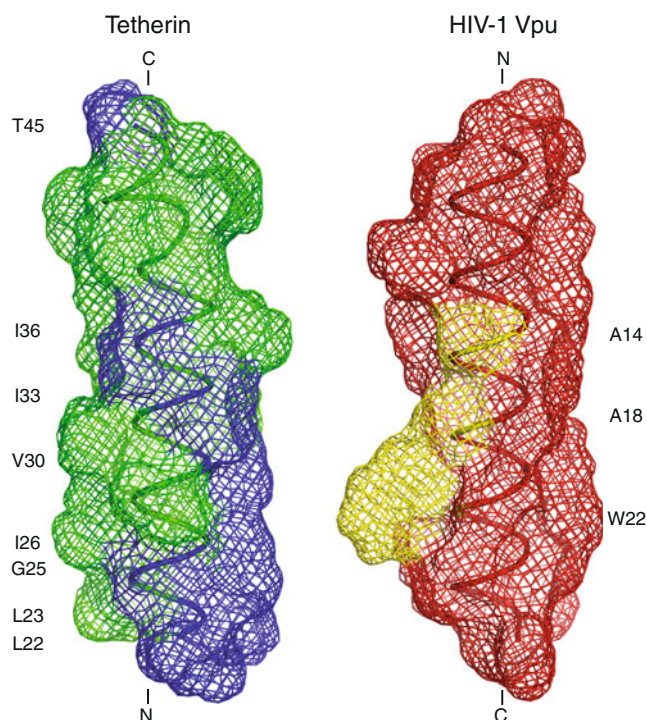


Fig. 2 Models of tetherin and Vpu transmembrane domains. The transmembrane domains of human tetherin (*left*) and HIV-1 Vpu (*right*) are illustrated in regard to their orientation in the membrane. Amino acids of the tetherin transmembrane known to impact on the interaction without abruption of tetherin function are highlighted in *blue* and indicated *left* to the model; amino acids are selected according to Gupta et al. (2009a), McNatt et al. (2009), and Rong et al. (2009). Amino acids that are involved in tetherin downmodulation from the cell surface are highlighted in *yellow* on the Vpu transmembrane model (Vigan and Neil 2010). Models were created with PyMol software on the basis of the sequences of human tetherin (GenBank ID NP_004326.1) and HIV-1 Vpu from viral clone pNL_4-3 (GenBank ID AAK08488.1)

of the prefabricated GPI anchor (Kupzig et al. 2003). Therefore, the mature tetherin is a heterogeneously glycosylated 160-amino-acid single pass transmembrane protein with a C-terminal GPI anchor incorporated into lipid rafts. The GPI anchor-mediated localization to lipid rafts promotes the clathrin-mediated endocytosis (Rollason et al. 2007). Removal

of GPI anchor abolishes the antiviral activity of tetherin (Neil et al. 2008; Perez-Caballero et al. 2009).

Models of “tethering” Several models have been proposed to illustrate how tetherin retains viral particles on the cell surface. These models are conceived on the basis of two key features of tetherin, dimerization across the ectodomain and insertion of the two termini into either the cellular plasma membrane or the membrane of viral particle. Since not only does tetherin act as a link to retain a virus particle to the cell surface but also tethers the viral particles to each other, it is believed that the N and the C termini of tetherin do not distinguish between the cellular and the viral membranes for insertion, which is not surprising given that viral membrane is derived from the cellular plasma membrane (Fig. 1b, c) (Neil et al. 2008; Perez-Caballero et al. 2009). Theoretically, a tetherin dimer may consist of two monomers that are positioned either in a parallel or in an antiparallel orientation. These two possibilities are now resolved by the crystal structures of tetherin ectodomains that are shown to form parallel dimers. Tetherin may thus function as a parallel homodimer to bridge cellular and viral membrane directly by insertion of one terminus into the cellular membrane and the other into the viral membrane (Fig. 1b). However, there is no conclusive evidence to rule out the possibility that tetherin dimers work by inserting the two termini of the same tetherin molecule into the same membrane (Fig. 1c).

Fate of tethered viral particles The tethered virions may be internalized and sent to late endosomes and lysosomes for degradation (Neil et al. 2007; Van Damme et al. 2008; Sato et al. 2009). It was recently shown that internalization and lysosomal degradation are promoted by the interaction of the cytosolic domain of tetherin with breast cancer associated gene 2 (Rabring7, ZNF364, RNF115) (Miyakawa et al. 2009). However, the molecular mechanisms behind this remain unknown. Furthermore, it is unclear whether internalized virions can cycle back to the cell surface if not degraded.

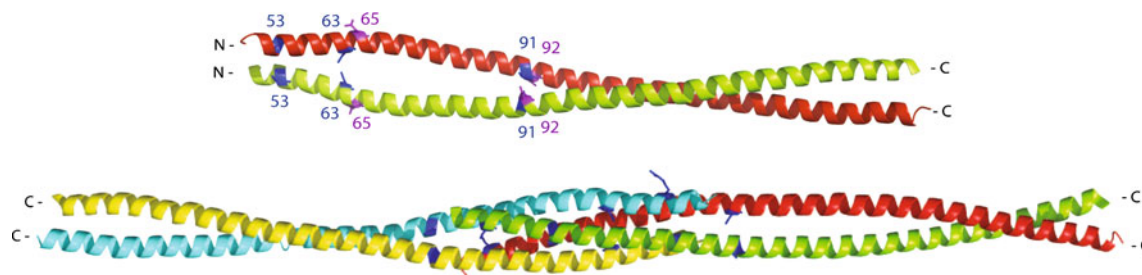


Fig. 3 Crystal structure of tetherin ectodomain. Shown are the crystal structures of a tetherin dimer (*top*) and tetramer (*bottom*). The disulfide bond building cysteine residues C₅₃C₆₃C₉₁ are highlighted in *blue* (*top* and *bottom*); N-glycosylation sites N₆₅N₉₂ are presented

in *purple* (*top*). Structures are based on the X-ray crystallography data from Schubert et al. (2010) (PDB ID: 3NWH) and were created using PyMol software. Structures of the ectodomains were created using PyMol software

By tethering the nascent virus to the cell surface, tetherin may affect cell-to-cell virus transmission. Cell-to-cell transmission takes place through the intercellular contact zones, termed virological synapses that temporarily connect the infected cell to the uninfected one (reviewed in Piguet and Sattentau 2004; Sattentau 2008; Martin and Sattentau 2009). Studies have been performed to assess the effect of tetherin on cell-to-cell transmission of HTLV-1 and HIV-1. Results showed that the tethered HTLV-1 particles on the cell surface promote direct cell-to-cell spreading of HTLV-1 (Pais-Correia et al. 2009). In the case of HIV-1, two studies reported a decreased cell-to-cell transmission as a result of the presence of tetherin at virological synapse (Casartelli et al. 2010; Kuhl et al. 2010b), whereas a third study observed the opposite effect (Jolly et al. 2010). Furthermore, differences were reported regarding the impact of tetherin on the infectivity of the transferred HIV-1 particles (Casartelli et al. 2010; Jolly et al. 2010; Kuhl et al. 2010b). Since cell-to-cell transmission is the predominant mode of HIV-1 spreading in cell culture and in secondary lymphoid tissues, clarification of the role of tetherin in cell-to-cell dissemination of HIV-1 will be of great importance.

In addition to holding viral particles to the cell surface, tetherin impairs the infectivity of Vpu-deficient HIV-1 particles that are released into the culture supernatant. Several groups have observed that tetherin reduces the levels of infectious Vpu-deficient HIV-1 particles to a more severe degree rather than decreasing the total amounts of virus particles as measured by viral CA(p24) or viral reverse transcriptase activity, which implicates an adverse effect of tetherin on the infectivity of progeny virions (Neil et al. 2008; Van Damme et al. 2008; Perez-Caballero et al. 2009; Sauter et al. 2009). Further studies revealed a defect in the morphology of cell-free HIV-1 particles that may result from incomplete processing of viral Gag proteins (Zhang and Liang 2010). The mechanism of this interference, however, is not yet clear.

How do viruses evade tetherin restriction?

Viruses have evolved different strategies to evade tetherin restriction. One common strategy that some viruses share is to shut down the interferon pathway such that tetherin is not induced for expression upon viral infection (reviewed in Versteeg and García-Sastre 2010). With this mechanism, not only tetherin but also other interferon-stimulated antiviral factors are not expressed in virus-infected cells. On the other hand, some viruses encode proteins that act as antagonists to counteract tetherin. The known tetherin antagonists include Vpu of HIV-1, envelope protein of HIV-2, SIVtan and Ebola virus, Nef of SIV, and K5 of KSHV. These viral proteins

overcome tetherin restriction by different mechanisms (summarized in Fig. 4 and Table 1).

HIV-1 Vpu HIV-1 Vpu is the most extensively studied tetherin antagonist among the others. Vpu is expressed from the vpu-env bi-cistronic viral RNA as a 16-kDa protein (reviewed in Bour and Strebel 2003; Malim and Emerman 2008). Although it is translated at the late stage of HIV-1 infection, Vpu is not found in virions. There exists a 27-amino-acid transmembrane domain at the N terminus of Vpu, followed by a 54-amino-acid cytoplasmic domain that consists of two α -helices (the number of amino acids refers to Vpu of the NL4-3 strain). It is worth noting that there are two serine residues at positions 52 and 56 that are located in the linker region of these two helices and are subject to phosphorylation. It is well documented that Vpu downregulates CD4 at the ER and thus prevents premature binding of viral gp160 with CD4. This function of Vpu initiates with recognition of the cytoplasmic tail of CD4 and recruitment of β -TrCP (a human beta transducin repeat containing protein) that is a key component of the E3 ubiquitin ligase complex (Skp1, Cullin1, and β -TrCP). The phosphorylated S52 and S56 residues of Vpu constitute the binding site for β -TrCP that subsequently causes poly-ubiquitination and proteasomal degradation of CD4.

The second prominent function of Vpu is to promote HIV-1 release by counteracting tetherin (Neil et al. 2008; Van Damme et al. 2008). One mechanism underlying this function of Vpu is to downregulate cell surface expression of tetherin and therefore removes tetherin from HIV-1 assembly and budding site on the plasma membrane (Van Damme et al. 2008; Perez-Caballero et al. 2009). The existence of additional mechanisms is also suggested by the observation of a significant level of tetherin incorporation into wild-type HIV-1 particles (Habermann et al. 2010). More importantly, Vpu enhances virus production in T-cell lines such as CEMx174 and H9 cells without reducing cell surface expression of tetherin (Miyagi et al. 2009).

Vpu may achieve tetherin downregulation from the cell surface by two mechanisms, depletion of the intracellular tetherin pool and sequestration of tetherin at the TGN. Vpu that is either produced from an expression vector or from HIV-1 proviral DNA is capable of diminishing the total amounts of tetherin in cells. This activity of Vpu relies on the recruitment of β -TrCP (Douglas et al. 2009; Mangeat et al. 2009; Mitchell et al. 2009). Mutating the S52/S56 residues of Vpu or depleting β -TrCP with small interfering RNA oligos significantly attenuates Vpu's antitetherin activity. In addition, expression of a dominant negative mutant of β -TrCP such as β -TrCP- Δ F also cripples the ability of Vpu to counteract tetherin. However, mutation of the only two lysine residues, the most common ubiquitin receptor, within the cytoplasmic tail of tetherin fails to

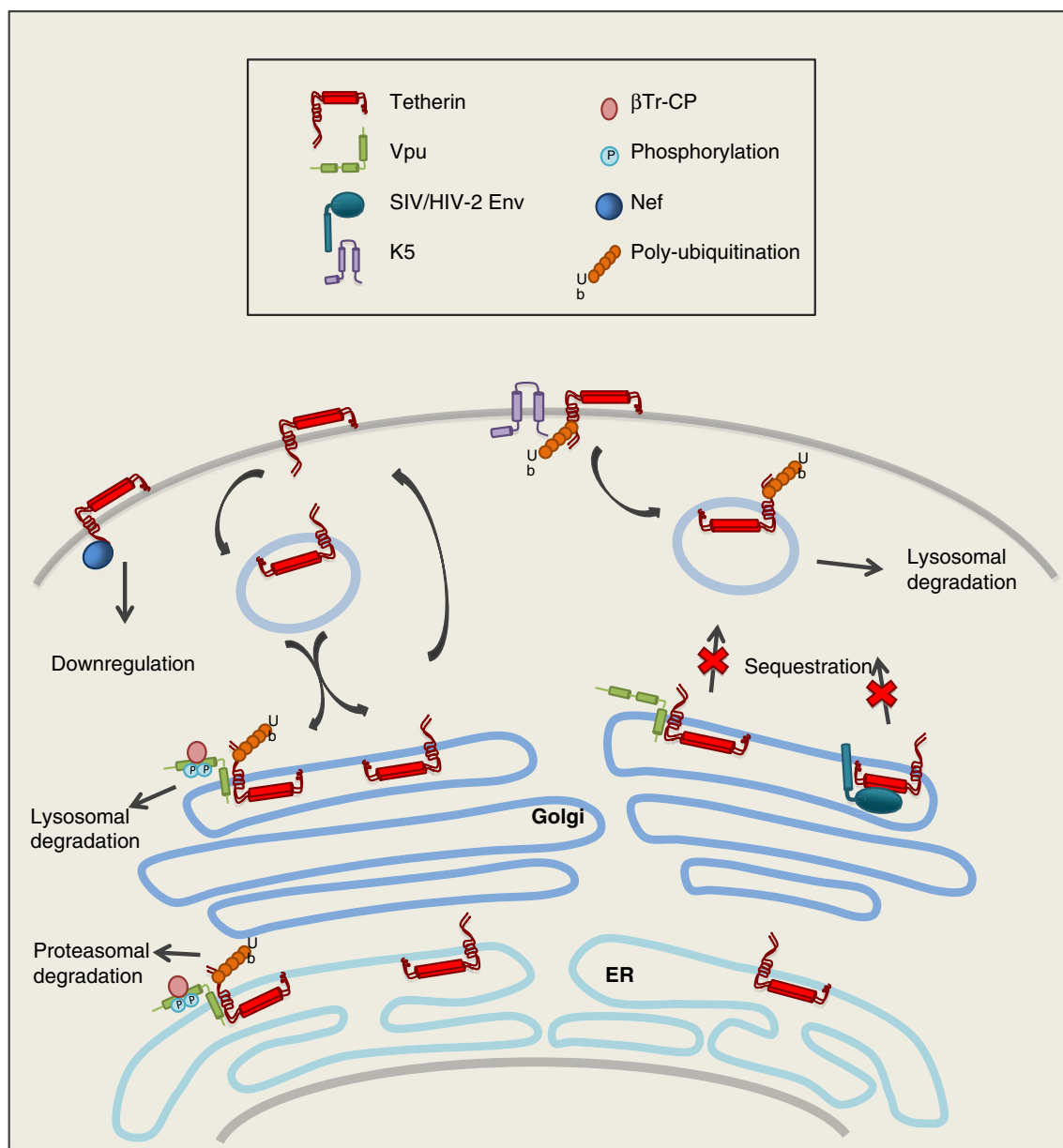


Fig. 4 Illustration of viral counter measures against tetherin. The normal cellular trafficking of tetherin is depicted from the endoplasmic reticulum (ER) to the Golgi apparatus, to the plasma membrane, and then through recycling endosomes. This trafficking is interrupted by several viral proteins that have been identified to antagonize tetherin. Vpu may sequester tetherin at the TGN or recruit β-TrCP to

ubiquitinate tetherin and target it for degradation via the lysosomal or proteasomal pathway. Nef interacts with the cytoplasmic tail of tetherin and triggers tetherin downregulation from the cell surface. SIV/HIV-2/Ebola envelope proteins may sequester tetherin at the TGN/perinuclear regions. K5 ubiquitinates tetherin and causes tetherin degradation in lysosomes

render the mutated tetherin resistant to Vpu-mediated degradation. The major Vpu-responsive site has now been mapped to the STS motif (positions 3 to 5) that undergoes Vpu/β-TrCP-dependent ubiquitination (Tokarev et al. 2010).

Vpu-mediated tetherin degradation may require either the proteasomal or the endosomal/lysosomal pathway. Interestingly, blocking either of these two degradation pathways by specific inhibitors in different cell lines

rescues Vpu-mediated tetherin degradation, albeit the extent of rescue varies due to specific experimental settings. Concanamycin A (ConA) is an inhibitor of the endosomal/lysosomal pathway and was observed to stabilize tetherin in the presence of Vpu (Douglas et al. 2009). Bafilomycin A1 (BafA1) reverses the cell surface and intracellular downregulation of tetherin by Vpu (Mitchell et al. 2009). Both BafA1 and ConA inhibit the endosomal vacuolar ATPase and prevent acidification of endosome and

Table 1 Summary of viral tetherin antagonists

Tetherin antagonists	Tetherin surface downregulation	Tetherin intracellular depletion	Target domain in tetherin	Intracellular relocalization of tetherin	Species specificity	References
HIV-1 Vpu	Yes	Yes	TM domain	TGN	Yes	Neil et al. (2008), Van Damme et al. (2008), Mitchell et al. (2009), Goffinet et al. (2009), Douglas et al. (2009), Mangeat et al. (2009), Iwabu et al. (2009), Dubé et al. (2010)
SIVcpz/gor Vpu	?	?	?	?	Yes	Sauter et al. (2009), Yang et al. (2010b)
SIVgsn/mus/mon Nef	?	?	Cytoplasmic tail (G/DDIWK)	?	Yes	Sauter et al. (2009), Yang et al. (2010b)
SIVmac/smm Nef	Yes	?	Cytoplasmic tail (GDDIWK)	?	Yes	Jia et al. (2009), Zhang et al. (2009)
SIVagmSab/Tan Nef	?	?	Cytoplasmic tail (DDICK)	?	Yes	Zhang et al. (2009)
HIV-2 Env	Yes	No	Ectodomain	TGN	No	Le Tortorec and Neil (2009), Jia et al. (2009), Hauser et al. (2010), Abada et al. (2005)
Ebola GP	No	No	?	?	No	Kaletsky et al. (2009), Lopez et al. (2010)
SIVtan Env	Yes	No	Ectodomain	Perinuclear region	No	Gupta et al. (2009b)
KSHV K5	Yes	Yes	Cytoplasmic tail(K18)	Late endosomes	Yes	Mansouri et al. (2009), Pardieu et al. (2010)

its maturation to late endosome. A study using a lysosomal protease inhibitor cocktail of leupeptin, pepstatin A, and E64d also supports the role of endosomal/lysosomal pathway in Vpu-mediated degradation of tetherin (Iwabu et al. 2009). Finally, knockdown of the μ 2 subunit of the AP-2 adaptor complex partially prevents the Vpu-induced tetherin downregulation (Mitchell et al. 2009).

MG-132 has been used in several studies as a proteasome inhibitor to prevent tetherin degradation by Vpu, but the results showed varying degrees of efficacy (Van Damme et al. 2008; Goffinet et al. 2009; Mangeat et al. 2009; Mitchell et al. 2009). It is noted that long-term treatment with MG-132 may deplete the intracellular pool of free ubiquitin and thus affect the ubiquitin-dependent degradation by either the proteasomes or the lysosomes (Van Damme et al. 2008; Douglas et al. 2009; Mitchell et al. 2009). Given the important role of β -TrCP and ubiquitination in the degradation of tetherin by Vpu, results obtained with MG-132 treatment alone may not be sufficient to distinguish between the roles of proteasomal or endolysosomal pathway in tetherin degradation. The use of the other proteasome inhibitors ALLN and clasto-lactacystin β -lactone (inhibitor of the 20S proteasome) increases the level of intracellular tetherin in the presence or

the absence of Vpu (Goffinet et al. 2009). Notably, both proteasome inhibitors abolish the ability of Vpu to rescue viral release in the presence of tetherin (Goffinet et al. 2009). It is possible that both the proteasomal and the endolysosomal pathways are involved in Vpu-mediated degradation of tetherin, albeit likely to varying degrees in different cell lines.

In addition to degradation, Vpu may disturb tetherin trafficking in cells and sequester tetherin away from the plasma membrane. Tetherin is normally localized to the plasma membrane and to, a less degree, in the perinuclear regions. Vpu causes a relocalization of tetherin from the plasma membrane to the TGN where tetherin is sequestered (Dubé et al. 2010). Results of immunoelectron microscopy showed an increased presence of tetherin at the early/recycling endosomes when Vpu is expressed (Habermann et al. 2010). It is conceivable that Vpu interacts with tetherin and diverts the trafficking of tetherin toward specific intracellular membrane compartments for sequestration or degradation.

Vpu likely recognizes tetherin through an interaction between the transmembrane domains of these two proteins. This scenario was first suggested by the finding that HIV-1 Vpu is unable to counteract tetherin from monkeys such as

African green monkey and Rhesus monkeys (Gupta et al. 2009a; McNatt et al. 2009; Rong et al. 2009). This resistance phenotype of monkey tetherin to HIV-1 Vpu was subsequently mapped to a few amino acid residues in the transmembrane domain of tetherin, which are divergent from their counterparts in the human tetherin transmembrane domain (Gupta et al. 2009a; Jia et al. 2009; McNatt et al. 2009; Zhang et al. 2009). A detailed mutagenesis analysis of the Vpu transmembrane domain also identified several residues that determine Vpu's tetherin antagonism activity (Vigan and Neil 2010). The Vpu mutants A14L, W22A, A14L/W22A, and, to a minor extent, A18L lost their ability to counteract tetherin (Vigan and Neil 2010). Further experiments showed that these Vpu mutants were crippled in downregulating tetherin from cell surface and were impaired in binding to tetherin. It is worth noting that the W22A mutant is also defective in downregulating CD4 from cell surface (Vigan and Neil 2010). In solid-state NMR structures of Vpu transmembrane multimers, A14, A18, and W22 line up on the outside-facing side of the helices (Park et al. 2003; Vigan and Neil 2010). These amino acid residues are likely involved in forming the interaction interface between the Vpu and the tetherin transmembrane helices (Fig. 2). In support of their importance in Vpu function, A18 and W22 are highly conserved in HIV-1 isolates that have retained the ability to antagonize tetherin. A detailed understanding of Vpu/tetherin interaction across their transmembrane domains requires further characterization of the involved amino acid residues in the tetherin transmembrane domain and structural determination of the Vpu/tetherin transmembrane domain complex. It also remains to be determined when and where Vpu initiates interaction with tetherin and how the Vpu/tetherin complex is retained at TGN and early/recycling endosomes.

SIV Nef SIV strains with the exception of SIV_{cpz}, SIV_{gor}, SIV_{gsn}, SIV_{mus}, and SIV_{mon} do not contain a Vpu gene (Gao et al. 1999; Courgnaud et al. 2002, 2003). Vpu of SIV_{gsn/mus/mon} antagonizes tetherin whereas SIV_{cpz/gor} Vpu does not overcome tetherin (Sauter et al. 2009). Therefore, SIV should have an alternative strategy to counteract tetherin. Indeed, Nef proteins from SIV strains including SIV_{mac}, SIV_{smm}, SIV_{blu}, and SIV_{agm} are able to overcome restriction by their respective species tetherin but are unable to overcome human tetherin (Jia et al. 2009; Zhang et al. 2009). Interestingly, Nef proteins from HIV-2 and HIV-1 are also able to counteract Old World monkey tetherins (Rhesus macaque and Sooty mangabey) but are less efficient than SIV Nef (Jia et al. 2009). The sensitivity determinant in simian tetherin to SIV Nef has been mapped to a five-amino-acid region (14-G/DDIWK-18) in the cytoplasmic domain, which is, however, absent in human

tetherin (Jia et al. 2009; Zhang et al. 2009). This is in contrast to the role of transmembrane domain of human tetherin in determining its sensitivity to HIV-1 Vpu (Jia et al. 2009; McNatt et al. 2009). Mutating the myristoylation site and cholesterol recognition motif in Nef impairs its tetherin antagonism activity (Jia et al. 2009; Zhang et al. 2009). Additionally, mutations that abrogate CD4 and CD28 downregulation activity of SIV_{mac239} Nef also diminish the antagonism of Rhesus tetherin, whereas MHC-I downregulation is not associated with tetherin-antagonizing function (Jia et al. 2009; Zhang et al. 2009). It is believed that SIV Nef protein counteracts simian tetherin by recognizing the "G/DDIWK" motif and down-regulating tetherin from the cell surface.

Envelope proteins of HIV-2, SIV, and Ebola virus The envelope glycoproteins (GP) of several viruses have been implicated in tetherin antagonism. A common feature among these tetherin antagonists is that they all seem to target the tetherin ectodomain and cause the relocalization of tetherin from the plasma membrane to the perinuclear region, a strategy that is similar to what Vpu employs to antagonize human tetherin.

HIV-2 Env Unlike HIV-1, HIV-2 does not encode a vpu gene. Instead, HIV-2 Env fulfills Vpu function to promote virus release (Bour et al. 1996, 2003; Bour and Strebel 1996, 2003). Further studies demonstrate that HIV-2_{RodA/Rod10} Env accomplishes this function also by counteracting tetherin (Le Tortorec and Neil 2009; Hauser et al. 2010). Yet this activity of HIV-2 envelope protein seems to be cell-type dependent, since the endogenous tetherin in HeLa cells but not the ectopically expressed tetherin in 293T cells is counteracted by HIV-2 Env. It is noted that HIV-1 Env and SIV_{mac} Env do not exhibit antitetherin activity (Le Tortorec and Neil 2009). The endocytic motif GYxxθ within the cytoplasmic tail as well as the extracellular motifs determines the antagonism activity of HIV-2 Env toward human tetherin (Le Tortorec and Neil 2009). In HIV-2_{Rod10} Env, a mutation in the membrane-proximal motif Y707 is sufficient to abrogate its antitetherin activity (Hauser et al. 2010). A point mutation A100D in the ectodomain of human tetherin is sufficient to render tetherin resistant to HIV-2 Env; however, both Ebola GP and HIV-1 Vpu were able to antagonize this mutant (Lopez et al. 2010). Tetherin was additionally shown to co-immunoprecipitate with HIV-2 Env likely through interaction between their extracellular domains, and tetherin was consequently downregulated from the cell surface (Le Tortorec and Neil 2009). HIV-2 Env does not cause intracellular depletion of tetherin but does lead to sequestration of tetherin at the TGN (Le Tortorec and Neil 2009). Additionally, Env from another isolate, HIV-2_{ROD14}, is unable to enhance viral release in cells

	cytosolic domain	transmembrane domain	ectodomain	
SMN (Chlorocebus aethiops)	MAPILYDYCKMPMDIDCKEDRDCKCKLAV--GILGLLVIVLLGVPLIFFIIKANSEA	QDGLRAVMECHNVTYLLQQLAEAEQGRDAAEQAVTCNQT	98	
AGM (Erythrocebus patas)	MAPILYDYCKMPMDIDCKEDGDKCKLAV--GILGLLVIVLLMGVPLIFFIIKASSEA	QDGLRAAMECRNVTYLLQQLAEAEQGRDAAEQAVTCNQT	98	
MAC (Macaca mulatta)	MAPILYDYCKMPMDIDCKEDGDKRCKLVV--GILGLLVIVLLGVPLIFFIIKANSEA	QDGLRAVMECRNVTYLLQQLAEAEQGRDAAEQAVTCNQT	98	
CPZ (Pan troglodytes)	MASTLYDYCRVPMDDIWKDGDGDKRCKLLLGIGILMLLIIVILGVPLIIIFTIKANSEA	CRDGLRAVMECRNVTHLLQQLTEAQKGFQDVEAQAATCNHTV	100	
Gorilla (Gorilla gorilla)	MASTLYDYCRVPMDDIWKDGDGDKRCKLLLGIGILVLLIIVILGVPLIIIFTIKANSEA	CRDGLRAVMECRNVTHLLQQLTEAQKGFQDVEAQAATCNHTV	100	
Human (Homo sapiens)	MASTSYDYCRVPM-----EDGDKRCKLLLGIGILVLLIIVILGVPLIIIFTIKANSEA	CRDGLRAVMECRNVTHLLQQLTEAQKGFQDVEAQAATCNHTV	95	
	1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100			
	ectodomain			
SMN (Chlorocebus aethiops)	MDLMSASDAEKAQGRKKVVEELEGITTLNHLKQDASAEVERLRRENQVLNVRIADTN-SASSQDS	SCAAEPPFLILLGLGALL	182	
AGM (Erythrocebus patas)	MALMASLDAEKAQGRKKVVEELEGITTLNHLKQDASAEVERLRRENQVLNVRIADTN-SASSQDS	SCAAEPPFLILLGLGALL	182	
MAC (Macaca mulatta)	MALMASLDAEKAHGKRVVEELEGITTLNHLKQDASAEVERLRRENHVLNARIADTN-SASPQDS	SCAAEPPFLILLGLSALL	182	
CPZ (Pan troglodytes)	MALMASLDAEKAQGGKKVVEELEEITTLNHLKQDASAEVERLRRENQVLSVRIADTKKYPSSQDS	SSAAAPQLLIVLLGLSALLQ	185	
Gorilla (Gorilla gorilla)	MALMASLDAEKAQGGKKVVEELEGITTLNHLKQDASAEVERLRRENQVLSVRIADTKKYPSSQDS	SSAAAPQLLIVLLGLSALLQ	185	
Human (Homo sapiens)	MALMASLDAEKAQGGKKVVEELEGITTLNHLKQDASAEVERLRRENQVLSVRIADTKKYPSSQDS	SSAAAPQLLIVLLGLSALLQ	180	
110.....120.....130.....140.....150.....160.....170.....180.....			

Fig. 5 Sequence alignment of primate tetherins. Aligned are tetherin amino acid sequences of selected simians from the Old World monkey lineage (SMN Sooty mangabey; AGM African green monkey; MAC Rhesus macaque) and hominid lineage (CPZ chimpanzee; gorilla; human). Highlighted are the domains that determine sensitivity to Nef (yellow), the cysteines that are involved in tetherin dimerization via

disulfide bonds (blue), and the GPI anchor attachment site (green). Alignment was created using ClustalX software; sequence files are derived from GenBank: SMN, ADI58600.1; AGM, ADI58599.1; MAC, ADI58602.1; CPZ, ADI58593.1; Gorilla, ADI58594.1; Human, NP_004326.1

expressing either human or Rhesus tetherin (Abada et al. 2005; Bartee et al. 2006; Jia et al. 2009). This defect seems to result from a point mutation at position 598 of HIV-2_{Rod14} Env (Abada et al. 2005; Bartee et al. 2006).

SIV Env The envelope glycoprotein from SIV_{tan} (Tantalus monkeys) antagonizes tetherin from human and different monkey species including Tantalus, Rhesus, and Sooty mangabey (Gupta et al. 2009b). SIV_{tan} Env appears to employ a similar antagonizing mechanism by downregulating the cell surface tetherin, but it does not cause depletion of intracellular tetherin. However, studies do show that SIV_{tan} Env causes relocation of human tetherin from the plasma membrane to the perinuclear region in 293T cells. A point mutation of the ectodomain of human tetherin (A100D) confers resistance to SIV_{tan} Env, suggesting the ectodomain as a possible target of SIV_{tan} Env (Gupta et al. 2009b). Whether other SIV strains also employ Env to counteract tetherin is currently unknown. At least, deletion of env in SIV_{mac} or SIV_{vag} does not increase virus sensitivity to tetherin (Jia et al. 2009; Zhang et al. 2009; Lim et al. 2010). It seems that SIV uses Nef protein more widely than Env protein to counteract tetherin.

Ebola GP Tetherin restricts the release of Ebola virus-like particles, and this restriction is overcome by Ebola GP (Kaletsky et al. 2009). It is striking that Ebola GP is able to circumvent tetherin from different species including human and murine as well as a series of functional tetherin derivatives including the active artificial tetherin (Lopez et al. 2010). A mutant GP that is retained at the ER by an ER retention signal is unable to overcome tetherin restriction, suggesting that correct localization of GP is necessary for its antagonism activity. GP antagonism of tetherin does not seem to affect intracellular levels of tetherin (Kaletsky et al. 2009; Lopez et al. 2010), nor does it require cell surface

tetherin downregulation (Lopez et al. 2010). Overall, Ebola GP appears to overcome a broad spectrum of tetherin via a mechanism that is distinct from that shared by the Env of HIV-2 and SIV_{tan}.

KSHV K5 KSHV expresses two RING-CH (MARCH) E3 ubiquitin ligases called K3/MIR1 and K5/MIR2. K5 has long been known to downregulate a number of cell surface proteins including MHC-I (Coscoy and Ganem 2000; Ishido et al. 2000; Stevenson et al. 2000). In a proteomics screening, tetherin was identified as a target of K5 downregulation from the cell surface (Bartee et al. 2006). Subsequent to the discovery of tetherin as a viral restriction factor, K5 was reported to overcome tetherin restriction and restore viral release (Mansouri et al. 2009; Pardieu et al. 2010). K5 exerts its antagonism activity by downregulating tetherin from the cell surface (Mansouri et al. 2009; Pardieu et al. 2010). This activity of K5 requires its RING domain that mediates the ubiquitination of the lysine residue at position 18 within tetherin cytoplasmic tail (Pardieu et al. 2010). This ubiquitinated tetherin is then sorted into CD63-positive late endosomal compartment for degradation (Pardieu et al. 2010). A dominant negative form of VPS4 prevents cell surface depletion of tetherin by K5 (Pardieu et al. 2010). Since this VPS4 mutant disrupts the multi-vesicular body (MVB) pathway, the results suggest that K5 redirects tetherin from the cell surface through the MVB pathway to late endosomes for degradation.

Tetherin is adapted to viral antagonists and shaped HIV evolution

Tetherin and other host cell restriction factors have co-evolved with viruses. The selective pressure from viruses has led to species specificity and diversity in restriction

factors with common ancestors; a sequence alignment of primate tetherins is shown in Fig. 5. Tetherin has also evolved under positive selection by viral antagonists (McNatt et al. 2009; Sauter et al. 2009; Lim et al. 2010). Evolutionary analysis revealed a positive selection at amino acid position 9 in the cytosolic domain of New World monkey tetherin; this was not seen with tetherins of Old World monkeys and hominids (Lim et al. 2010). Instead, tetherin amino acid position 17 has undergone positive selection in Old World monkeys and hominids, in addition to a deletion of five amino acids surrounding this position (14-DDIWK-18) in human tetherin (Lim et al. 2010). It is believed that the positive selection has been driven by an ancient Nef-like antagonist of tetherin (Lim et al. 2010). Interestingly, Vpu does not seem to have impacted on tetherin evolution in Old World monkeys or hominids, but this might be due to the short period of time since the gain-of-function evolution in Vpu (Lim et al. 2010).

HIV-1 was originated by cross-species transmission of SIV from chimpanzees to humans (Gao et al. 1999). SIV_{cpz} is considered to have evolved from a recombination of two SIV strains, SIV_{gsn} and SIV_{rcm}. SIV_{gsn} but not SIV_{rcm} encodes Vpu (Courgnaud et al. 2002, 2003; Dazza et al. 2005). SIV_{cpz} obtained Vpu from SIV_{gsn} and Nef from SIV_{rcm}. It is speculated that, in the original SIV_{cpz}, Vpu and Nef proteins had only little antitetherin capacity (Sauter et al. 2009; Yang et al. 2010b). Over time, SIV_{cpz} Nef evolved to become the primary tetherin antagonist, while Vpu maintained the capacity to downmodulate CD4 from the cell surface (Sauter et al. 2009; Yang et al. 2010b). When SIV_{cpz} crossed the species barrier to infect humans, Nef was unable to antagonize human tetherin due to the lack of the Nef-sensitive ¹⁴DDIWK₁₈ site. Vpu subsequently (re) gained its tetherin-antagonizing function (Sauter et al. 2009; Zhang et al. 2009; Lim et al. 2010). However, only the Vpu of pandemic HIV-1 group M efficiently antagonizes human tetherin whereas Vpu of group N and O is a poor tetherin antagonist (Sauter et al. 2009). This suggests that the extent of Vpu adaptation to antagonize human tetherin influences the pathogenicity of HIV-1.

In contrast to SIV_{cpz}, the SIV_{smm} strain that gave rise to HIV-2 lacks vpu (Gao et al. 1992; Chen et al. 1997). HIV-2 overcame human tetherin restriction through evolving its envelope protein into a tetherin antagonist (Le Tortorec and Neil 2009). This adaptation, however, might involve a fitness cost, which is reflected by the low infectivity of HIV-2 when compared to HIV-1. Nonetheless, this flexibility in viral strategy to antagonize tetherin reflects the intense selective pressure exerted by tetherin during the adaptation of the HIV-1 and HIV-2 precursor viruses to the new host. Considering the high conservation of tetherin in various species and its restrictive nature over a wide range of enveloped viruses, the example of shaping HIV-1 and

HIV-2 evolution suggests a highly active role of tetherin-mediated innate immunity against cross-species transmission of viruses beyond HIV (McNatt et al. 2009; Sauter et al. 2009; Lim et al. 2010; Yang et al. 2010b).

Concluding remarks

Since the discovery of the potent antiviral activity of tetherin, efforts are already underway to exploit Vpu/tetherin interaction as a new target to develop novel anti-HIV-1 therapeutics such as small compounds that block Vpu function and thus expose HIV-1 to tetherin restriction. Similar approaches have been proven successful in the development of small molecule inhibitors of Vif/APOBEC3G interaction (Nathans et al. 2008; Cen et al. 2010). It has already been reported that a Vpu-targeting drug BIT225 inhibited the release of HIV-1 from macrophages (Khouri et al. 2010). However, protease inhibitors, which modulate transmembrane transporters and thereby might also interfere with Vpu, proved not to impact on tetherin cell surface expression or Vpu-mediated downmodulation of tetherin (Kuhl et al. 2010a).

It is noted that the anti-HIV-1 activity of tetherin and the Vpu antagonism have been investigated thus far mainly in cultured cell lines including HeLa, 293T, COS-7, HT1080, CEMx174, H9, and SupT1 cells. The results thus obtained need to be verified by experiments in primary human immune cells such as CD4⁺ T cells and macrophages partially because some observations have been cell-type-specific. For instance, although HIV-1 replication is Vpu-responsive in CEMx174 and H9 cells, the cell surface level of tetherin is not affected by Vpu expression as opposed to the downregulation of cell surface tetherin by Vpu in HeLa and 293T cells (Miyagi et al. 2009; Van Damme et al. 2008). The physiological role of tetherin in the pathogenesis of HIV-1 and other enveloped viruses also remains to be determined in animal models. Although tetherin potently blocks the release of virus particles from cell surface and thus restricts dissemination of free virus particles, further investigation is needed to clarify its role in cell-to-cell virus transmission that is much more efficient than transmission by free virus particles.

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Conflict of Interest There is no conflict of interest for any of the authors.

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